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Mechanisms of radiation-induced bone loss and effects on prostate cancer bone metastases

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14. ABSTRACT Patients with prostate cancer frequently receive radiation therapy. Although radiation therapy is effective for the treatment of primary tumors, bystander bone absorbs approximately half of the radiation dose and thus may cause adverse radiation-induced effects at skeletal sites. Indeed, within five years of radiation treatment at the pelvic region (cervical, rectal or anal cancer), the risk of hip fracture increases by up to 20% in cancer patients relative to the general population. During the reported project period, a dose-response and time-course study using <i>in situ</i> bone calvarial assays demonstrated that radiation exposure (6-10Gy) causes osteocyte apoptosis one week following exposure. Osteoblast apoptosis was not observed during the time course (1-10 days) at any dose (2-10Gy). Next, using a single-limb therapeutic radiation model, I examined the direct and indirect effects of radiation on bone <i>in vivo</i> . Our data demonstrate that a relatively low dose of site-directed radiation (2Gy) caused direct and systemic bone loss in C57Bl/6 mice relative to previously untested sham-irradiated controls and that osteoclastogenesis and marrow adiposity were drastically increased one week following radiation exposure at site of direct irradiation. Assayed sera from irradiated mice revealed no change in classic inflammatory pro-resorptive cytokines (IL-1 β , IL-6, IL-17, TNF- α); however, we did observe a significant decline in anti-inflammatory cytokines (IL-3, IL-4, IL-10 and RANTES/CCL2) relative to sham-irradiated controls. Using dynamic histomorphometry, mineralized surface area of bone at sites of irradiation was reduced, with no change in the number of osteoblasts, indicating that osteoblast function may be impaired by irradiation. We therefore conclude that bone loss resulting from radiation exposure <i>in vivo</i> can be attributed to an increase in osteoclastic bone resorption and reduced mineralization of bone surfaces. Finally, during the reported project period, the single limb irradiation model was successfully reproduced in the Balb/c nu/nu strain of mouse required for our prostate cancer studies; thus, we will now commence our final proposed tasks wherein we plan to evaluate how radiation-induced bone loss affects the progression of prostate cancer bone metastases. Theses studies will provide new insight into tumor biology and the impact of insults to the bone microenvironment on prostate cancer bone metastasis.					
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INTRODUCTION

Patients with tumors in the pelvic region (e.g., prostate cancer) frequently receive radiation therapy to treat the primary tumor [1]. Although radiation therapy is effective for the treatment of primary tumors, bystander bone absorbs approximately half of the radiation dose and thus may cause adverse radiation-induced effects at skeletal sites, including bone loss and increased fracture risk at the hip [2-4]. Indeed, the risk of hip fracture within five years of radiation treatment at the pelvic region (cervical, rectal or anal cancer) increases by up to 20% in cancer patients relative to the general population [5-7]. Hip fracture causes considerable morbidity and mortality to cancer patients during the course of treatment and poses a significant threat to overall patient outcome. It has long been recognized that radiation induces bone loss and recently it has been demonstrated that osteoclast activity and bone resorption increase significantly 24 hours following radiation exposure [8,9]. The mechanism of this rapid increase in osteoclastogenesis is unknown. It has become increasingly clear that osteocytes are active regulators of bone turnover and that osteocyte apoptosis precedes differentiation of osteoclasts and increased bone resorption [10,11]. Moreover, previous studies have reported that radiation exposure causes osteocyte apoptosis [12,13]. We therefore hypothesized that osteocyte apoptosis is a key mechanism involved in the induction of osteoclastic bone resorption and bone loss following radiation therapy. *In vitro* studies presented here were therefore designed to elucidate direct and indirect effects of ionizing radiation on bone cells (osteoblasts, osteocytes, osteoclasts, bone marrow cells) in dose- and time-dependent fashion. Using an established single-limb model of radiation exposure, *in vivo* studies reported here examine the direct and indirect effects of radiation on bone. Finally, preliminary data that we have obtained demonstrate that an elevated state of high bone turnover can increase cancer metastasis to bone. We therefore hypothesized that radiation-induced bone loss could increase the progression of prostate cancer bone metastases in mice. Using an established murine model of prostate cancer bone metastasis, we will assess the effects of single-limb irradiation on prostate cancer cell metastasis to bone. Results obtained from this fellowship training grant will increase understanding of a relatively common, but unstudied area that affects patients with prostate cancer, and may eventually contribute to implementation of therapeutic interventions that can reduce fracture risk in patients receiving radiation therapy. Finally, the studies will provide new insight into tumor biology and the impact of insults to the bone microenvironment on prostate cancer bone metastasis.

BODY

Task 1: Determine time- (6–48 hours) and dose- (0-10Gy) dependent effects of radiation on apoptosis of the osteocyte cell line and freshly isolated osteocytes (vs. osteoblasts) [months 1-8]

a. Perform apoptosis assays (TUNEL and Trypan Blue staining, AnnexinV-EGFP/Propidium flow cytometry) [months 2-4] on:

1. Osteocyte-like cell line, MLO-Y4 (vs. MC3T3-E1 osteoblast cell line)
2. Isolated osteocytes (vs. osteoblasts) from mouse calvariae

Animal Usage: 5 balb/c mice; Cells: MLO-Y4, MC3T3-E1 cells + freshly isolated osteocytes and osteoblasts

Completion of this task will reveal whether MLO-Y4 osteocytes and freshly isolated osteocytes are sensitive to the effects of radiation. *Collaborator: Dr. Bellido*

a.1. Complete (previously presented)

a.2. During this award period, I continued working with the neonatal calvarial tissues and examined the effects of radiation on late phase apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), in order to determine a dose-response and time-course for DNA damage indicative of osteocyte and osteoblast apoptosis following irradiation. Osteocyte apoptosis was observed on days seven and ten post-irradiation and only at radiation doses of 6Gy or higher (**Figure 1A**). Surprisingly, osteoblast apoptosis was not detected over time at doses as high as 10Gy (**Figure 1B**). These results confirm that osteocytes are sensitive to radiation exposure at 6Gy or higher and that osteoblast cells are less susceptible to radiation-induced DNA damage.

Task 2: Determine effect of osteocyte apoptosis on osteoclastogenesis [months 6-9]

- a.** Establish a new *in vitro* osteoclast Assay using RAW264.7 osteoclast progenitor (Corning Inc.) [months 6-7].
- b.** Test osteoclastogenic capability of apoptotic bodies and medium from osteocytes (Task 1) [months 8-9].

Completion of this task will reveal whether radiation-induced apoptosis of osteocytes can induce osteoclastogenesis. *Cells lines used: RAW264.7, MLO-Y4, MC3T3-E1.*

a-b. Complete (previously presented)

Task 3: Test effects of single limb exposure (distal femur and total tibia) to 2Gy [months 6-10]

- a.** Perform histomorphometry (OC, OT, OB counting) and *in vivo* bone imaging [months 6-10].
- b.** Determine apoptosis of bone cells (OT, OB & OC) by quantifying TUNEL staining [months 6-10].

Animal Usage: 30 balb/c mice

Completion of this task will reveal whether osteocyte apoptosis is associated with induced osteoclastic bone resorption.

a. The novel discovery that non-irradiated contralateral limbs lose a significant amount of bone volume (BV/TV) relative to baseline measurements in our previous studies suggested that radiation therapy may cause systemic bone loss. I followed up on these studies with additional *in vivo* work to elucidate whether systemic changes resulting from focal irradiation could cause bone loss in non-irradiated tissues. In order to test this new and important hypothesis, an *in vivo* study was designed wherein C57Bl/6 mice were sorted randomly into two treatment groups ($n=15$) and assessed for baseline bone volume by μ CT. Mice in group A were anesthetized with isoflurane and irradiated on the right limb with a nominal surface dose of 2Gy using a therapeutic X-ray Machine (XRAD 320 Precision) operating at an effective dose rate of 1.5946 Gy/min. Group B was sham-irradiated (0Gy). Seven days later, a final *in vivo* μ CT scan was performed in all groups, mice were euthanized by cervical dislocation, blood was collected and bone and soft tissues were fixed and saved for bone histomorphometry and histological analyses. Bone volume fraction (BV/TV) declined significantly in irradiated and non-irradiated contralateral tibiae relative to sham-irradiated control mice as assessed by ANOVA at the final timepoint (**Figure 2A**), and these effects were further confirmed using paired analyses for each individual bone (**Figure 2B-D**). Serum samples collected at baseline (prior to irradiation) and seven days post-irradiation were assayed by Bio-plex® so that multiple cytokines could be detected in one sample. Surprisingly, classic pro-inflammatory and pro-bone-resorptive cytokines (IL-1 β , IL-6, IL-17 and TNF- α) did not change or declined in both control and irradiated animals (**Figure 3A**), indicating that systemic changes in inflammatory factors that typically stimulate bone resorption may not be responsible for radiation-induced bone loss in contralateral limbs. Anti-inflammatory and pro-bone-formative cytokines (IL3, IL-4, IL-10 and RANTES/CCL2) did not change over time in control animals (**Figure 3B**), however, these cytokine levels were significantly reduced in irradiated mice (**Figure 3B**). These data indicate that changes in systemic bone-formative factors could be responsible for impaired bone mineralization following radiation exposure. Osteoclastogenesis and marrow adiposity significantly increased in irradiated bone (**Figure 4A,C**), but no changes in osteoblast numbers were detected (**Figure 4B**). Despite no change in osteoblast numbers, osteoblast function may be impaired by radiation, as dynamic histomorphometry measurements showed a significant decreased in mineralized bone surface in irradiated femora in the trabecular bone compartment (**Figure 5B,C**) but no change in the mineralized apposition rate (MAR) of bone (**Figure 5A**). These results are concurrent with previous studies presented for Task 3, however these new results contain critical data obtained from naïve sham-irradiated age-matched control mice, a key control group required for the manuscript publication described in Task 11.

b. Complete (previously presented)

Task 4: Determine if blocking osteocyte apoptosis will block bone loss associated with radiation [months 10-16]

- a.** Single limb exposure of 2Gy [month 10].
- b.** Treat mice with two apoptosis inhibitors: **i)** A caspase inhibitor that has been used in mice to inhibit osteocyte apoptosis induced by bone over-loading; and **ii)** a bisphosphonate that inhibit osteocytes apoptosis, but that does not affect osteoclasts (IG9402). Zoledronic acid will be used as positive control for inhibition of apoptosis and also inhibition of resorption [month 10].
- c.** Perform *in vivo* bone imaging and histomorphometry and assay apoptosis of bone cells (osteocyte, osteoblast and osteoclast) by quantifying TUNEL staining [months 10-14].

Animal Usage: 40 balb/c mice

Completion of this task will reveal 1) whether radiation-induced bone loss is mediated via osteocytes, and 2) what treatment is most effective in inhibiting the radiation-induced bone loss. *Collaborators/consultants: Dr. Mendonca, Dr. Bateman, Dr. Bellido*

a-c. Due insufficient *in vitro* and *in vivo* evidence that radiation-induced osteocyte apoptosis is the main driver of osteoclastogenesis and bone loss following radiation exposure, we have elected to further pursue the mechanism of systemic bone loss by assaying serum levels of cytokines in our animals, as described and presented in Task 3.

Task 5: Image calvarial osteocytes in real-time after single dose exposure of 2 Gy [months 6-12]

A single dose of 2Gy will be given to the calvariae of transgenic mice, in which GFP is specifically expressed in osteocytes (DMP1-EGFP mice).

a. Develop intravital multiphoton fluorescence microscopy (IVFM) for real-time imaging of osteocytes in calvariae of transgenic mice using i) GFP to identify osteocytes; ii) Hoechst to identify apoptotic cells; iii) Texas red to identify vasculature. A time course of morphological changes will be monitored in the same animals at every 2 days during the first week post radiation and then weekly for up to 4 weeks. Animals will be euthanized 7, 14, 21 or 28 days after initiation of the experiments and we will [month 6-10]. Quantify *in vivo* + *ex vivo* osteocyte apoptosis, determined as green cells that contain Hoechst nuclei that are condensed (apoptotic) [month 6-7].

b. Perform histomorphometry (OC, OT, OB counting) and *in vivo* bone imaging [months 10-14]. *Ex vivo* determination of apoptosis by quantifying TUNEL staining [months 10-14].

Animal Usage: 24 transgenic DMP1-GFP mice, in which GFP is specifically expressed in osteocytes.

Completion of this task will reveal the time-dependent effects of a single dose of radiation of 2Gy on osteocyte apoptosis.

Collaborators/consultants: Dr.Mendonca, Dr.Bateman, Dr.Bellido, Dr. Carlesso

a-b. Due insufficient *in vitro* and *in vivo* evidence that radiation-induced osteocyte apoptosis is the main driver of osteoclastogenesis and bone loss following radiation exposure, we have elected to further pursue the mechanism of systemic bone loss by assaying serum levels of cytokines in our animals, as described and presented in Task 3.

Task 6: Real-time imaging of the effects of apoptosis inhibitors on osteocytes in calvariae [months 12-15]

a. Set up radiation model of real-time imaging as described in Task 5, and treat mice with apoptosis and bone resorption inhibitors as described in Task 4 [month 12].

b. Assess the effects of treatment on osteocyte apoptosis with IVFM, histomorphometry, by quantifying TUNEL staining [months 12-15].

Animal Usage: 40 balb/c mice

Completion of this task will show a time-course of the inhibitory effects of treatments on osteocyte apoptosis.

Collaborators/consultants: Dr. Mendonca, Dr. Bateman, Dr. Bellido, Dr. Carlesso

a,b. This task has not been completed due to the lack of evidence that osteocytes are the main drivers of osteoclastic bone resorption during radiation-induced bone loss. Additionally, the technician who specializes in real time intravital multiphoton fluorescence microscopy (IVFM) has left Indiana University. Alternatively, I focused my work on the *ex vivo* evaluation of osteocyte apoptosis in calvariae by TUNEL stain in the dose- and time-response experiments described in Task 1.

Task 7: Determine if radiation induces growth of prostate cancer bone metastases with a lytic (PC-3) or mixed (C4-2B) phenotype, and whether osteocyte apoptosis blockers can prevent this [months 5-18]

a. Intracardiac injection of C4-2B/IFP or PC-3/IFP prostate cancer cells, 1 week after single limb exposure of 2Gy [month 10].

b. Treat mice with apoptosis inhibitors *as described in Task 4* [month 10].

c. Assess tumor burden and bone loss by *in vivo* near-infrared imaging (Optix MX3), bone histology and histomorphometry (OC, OT, OB counting) [months 12-16].

Animal usage (cell line used): 40 balb/c nu/nu mice (PC-3 pcc) + 40 SCID mice (C4-2B pcc)

Completion of this task will reveal (1) whether radiation induces formation and growth prostate cancer bone metastases with lytic or mixed phenotype, and (2) what treatment is most effective in inhibiting it.

Collaborators/consultants: Dr.Mendonca, Dr.Bateman, Dr.Bellido

a-c. During this period, I have completed a critical study applying the single limb irradiation model to young (four-week) male Balb/c nu/nu mice. The purpose of this experiment is to verify that the effects of radiation-induced bone loss are

similar in different strains of mice (C57Bl/6 mice versus Balb/c nu/nu) and in different stages of development (20-weeks versus four-weeks of age). These experiments are critical because the Balb/c nu/nu athymic immune-deficient mouse strain is the strain required for our prostate cancer bone metastasis model described in Tasks 7 and 8. Similar to experiments presented earlier using C57Bl/6 mice, bone μ CT analyses have been performed on irradiated and non-irradiated limbs at baseline and seven days post-irradiation, and compared to sham-irradiated Balb/c nu/nu mice in order to confirm that the single site model of therapeutic irradiation can be replicated 1) in male immune-suppressed Balb/c nu/nu mice and 2) in younger four-week animals.

In order to accomplish this, an *in vivo* study was designed wherein four-week male athymic Balb/c nu/nu mice were sorted randomly into two treatment groups ($n=15$) and assessed for baseline bone volume fraction (BV/TV) by μ CT. Mice in group A were anesthetized with isoflurane and irradiated on the right limb with a nominal surface dose of 2Gy using a therapeutic X-ray Machine (XRAD 320 Precision) operating at an effective dose rate of 1.5946 Gy/min. Group B was likewise anesthetized, and placed in the X-ray cabinet for the same duration as a sham control (0Gy). Seven days later, a final *in-vivo* μ CT scan was performed in all groups, mice were euthanized by cervical dislocation, blood was collected and bone and soft tissues were fixed and saved for bone histomorphometry and histological analyses. Bone volume fraction (BV/TV) declined significantly in irradiated and non-irradiated contralateral tibiae relative to sham-irradiated control mice (**Figure 6A,B**). Evidence of systemic bone loss resulting from single site-directed radiation was further corroborated in this experiment, and a trend for reduced bone volume fraction was clearly seen in the lumbar vertebrae of irradiated mice (**Figure 6C**), further supporting the notion that single site irradiation can cause bone loss at non-irradiated sites. The single limb irradiation model is now established in male four-week Balb/c nu/nu mice, and next I will test the effects of radiation on prostate cancer metastasis progression as described in this task [months 18-22]. Because we have found no evidence that osteocytes are the primary cell type responsible for radiation-induced bone loss, osteocyte apoptosis blockers will not be utilized.

Task 8: Determine if radiation induces growth of blastic prostate cancer bone metastases, and whether osteocyte apoptosis blockers can prevent this [months 12-18]

- a. Intra-bone injection with LuCaP 23.1 (AR+, PSA+) prostate cancer cells in both tibiae [month 12].
- b. Single limb exposure with 2Gy [month 12].
- c. Treat mice with apoptosis inhibitors *as described in Task 4* [month 12].
- d. Assess tumor burden and bone loss by radiographic and histomorphometric analyses [months 12-18].

Animal Usage (cell line used): 40 SCID mice (LuCaP23.1 prostate cancer cells)

Completion of this task will reveal 1) whether radiation induces growth of prostate cancer cells that yield a blastic phenotype in bone, and 2) what treatment is most effective in inhibiting it.

Collaborators/consultants: Dr.Mendonca, Dr.Bateman, Dr.Bellido

a-d This task will be completed simultaneously with Task 7 in Balb/c nu/nu mice now that the single-limb irradiation model has been replicated in Balb/c nu/nu mice [months 20-24]. Because we have found no evidence that osteocytes are the primary cell type responsible for radiation-induced bone loss, osteocyte apoptosis blockers will not be utilized.

Task 10: Write a review of the literature [months 12-18]

Completion of this task will result in a peer-reviewed publication in a high impact journal.

A final review article will be written upon completion of all our studies [Timeframe: months 20-24].

Task 11: Prepare manuscript [months 12-24]

Completion of this task will result in a peer-reviewed publication in a high impact journal.

Currently, I am preparing results from Tasks 1-6 for publication in the *Journal of Bone and Mineral Research*, with anticipated submission occurring in month 19. This manuscript entitled "Single site irradiation has direct and systemic effects on bone in mice," contains all *in vitro* data obtained to date, and all data pertaining to the single limb irradiation of C57Bl/6 mice. A final manuscript describing the effects of single limb irradiation in Balb/c nu/nu mice on bone and on the progression of prostate cancer bone metastases will be written upon completion of Tasks 7-8 [month 24].

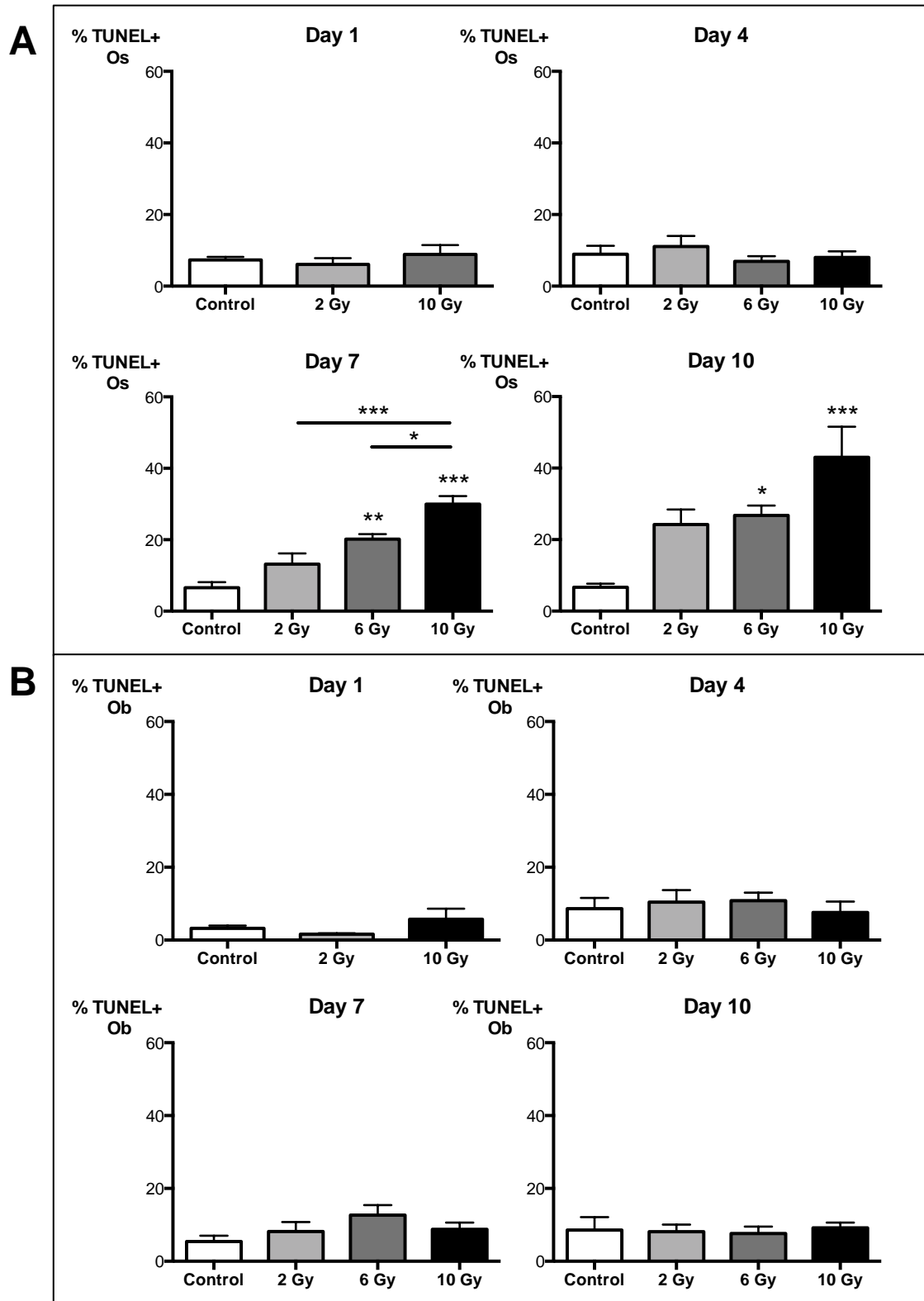


Figure 1. Osteocyte (Os) apoptosis increases dose- and time-dependently in *ex vivo* irradiated neonatal calvarial tissue. Neonatal calvarial tissue was harvested and cultured in DMEM modified media with 10% FBS, irradiated at varying doses on day 0 (0Gy-10Gy) and cultured for 1-10 days. Effects of radiation on (A) osteocyte and (B) osteoblast (Ob) were quantified by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by one-way ANOVA).

Single limb irradiation in male C57BL/6

Bone volume fraction (BV/TV) at the proximal tibia

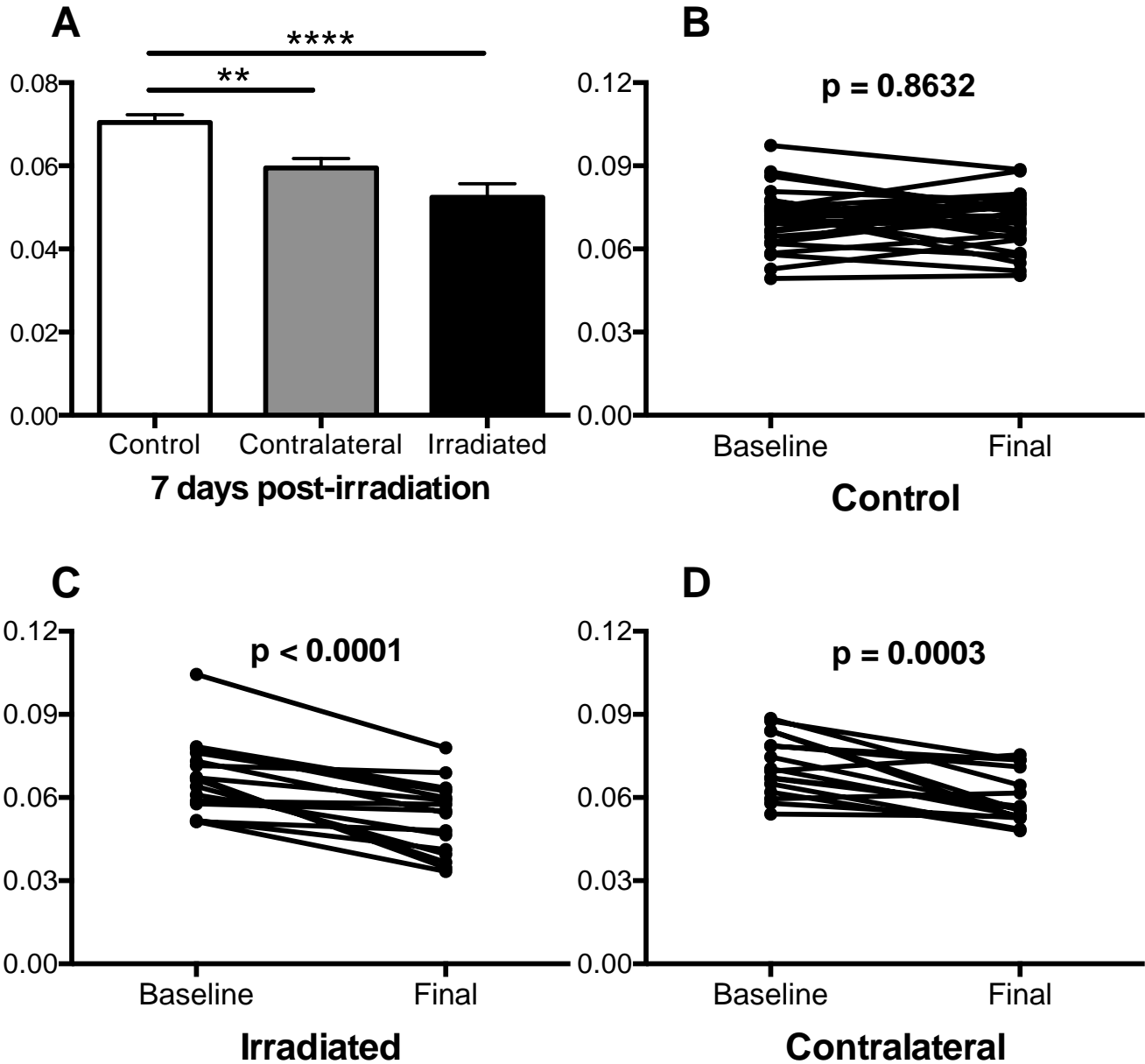


Figure 2. Single limb irradiation causes bone loss in irradiated and non-irradiated contralateral tibiae relative to naïve control bone. Twenty-week male C57BL/6 mice were irradiated (2Gy) at the right hindlimb and bone volume fraction (BV/TV) was measured in (A,B) naïve control sham-irradiated tibiae, (C) directly irradiated tibiae and (D) non-irradiated contralateral tibiae by bone microcomputed tomography at baseline and seven days post-irradiation (**p<0.01; ***p<0.001 by one-way ANOVA).

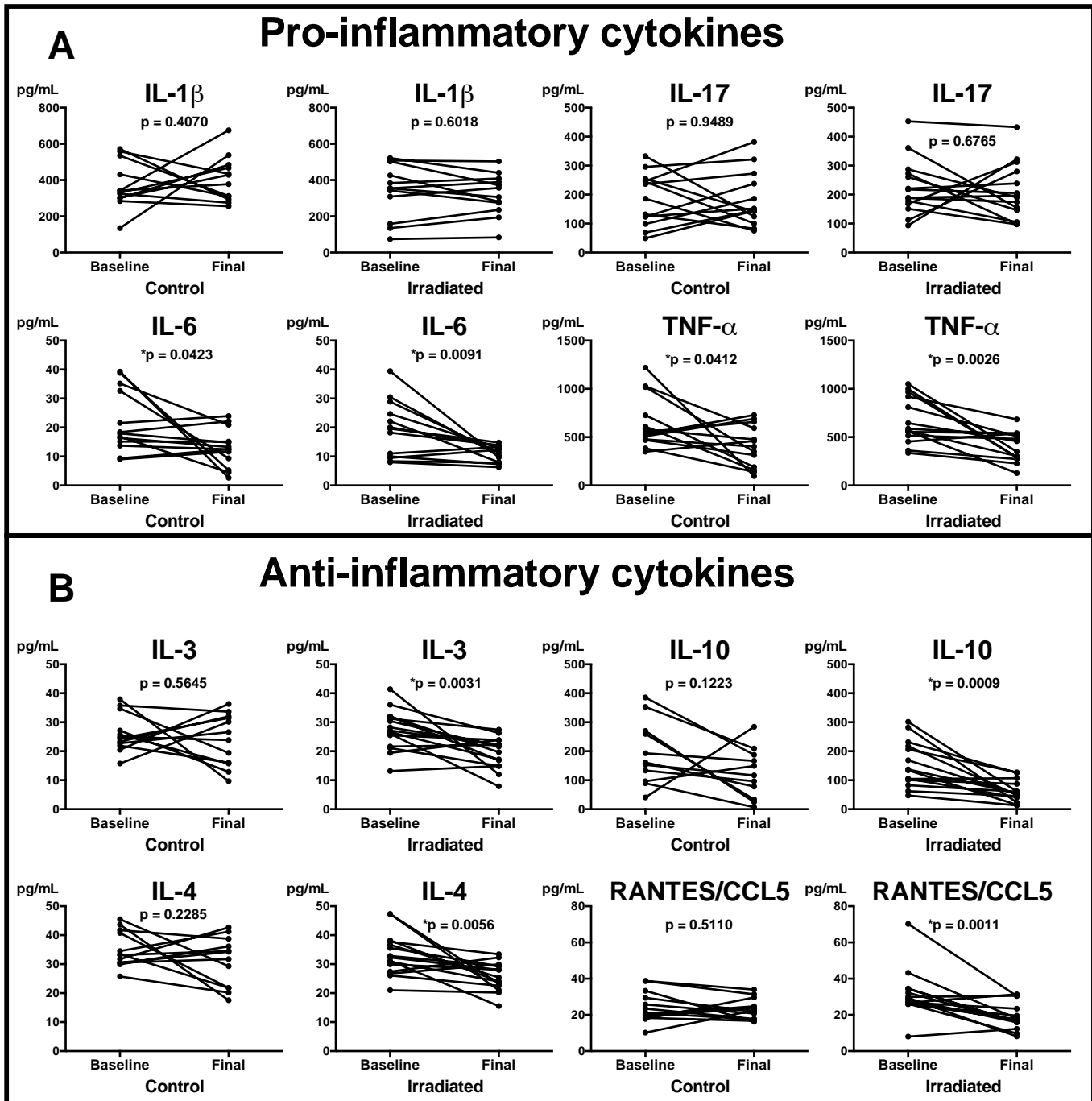


Figure 3. Single limb irradiation causes a decrease in systemic anti-inflammatory and bone-formative cytokine levels. Twenty week male C57Bl/6 mice were irradiated (2Gy) at the right hindlimb and serum was collected at baseline and seven days post-irradiation. (A) Pro-inflammatory cytokines and (B) anti-inflammatory cytokine levels were measured by Bio-plex® assay (paired t-test analyses).

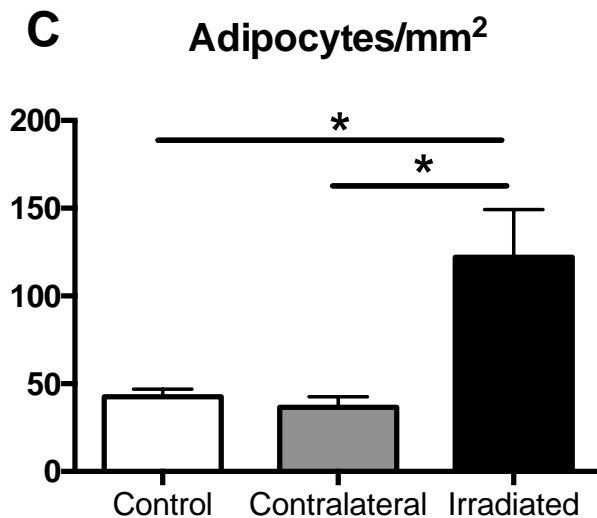
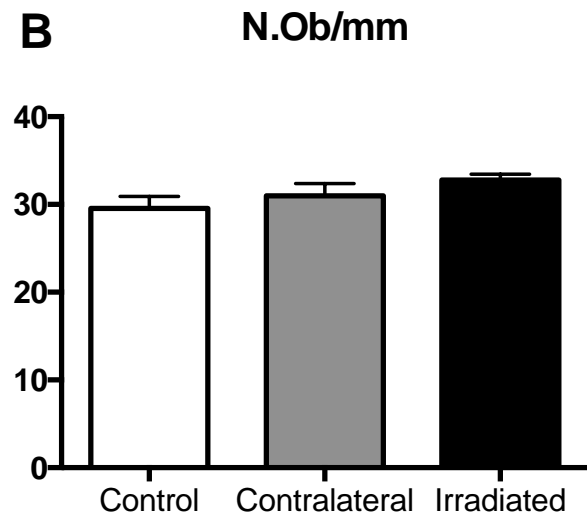
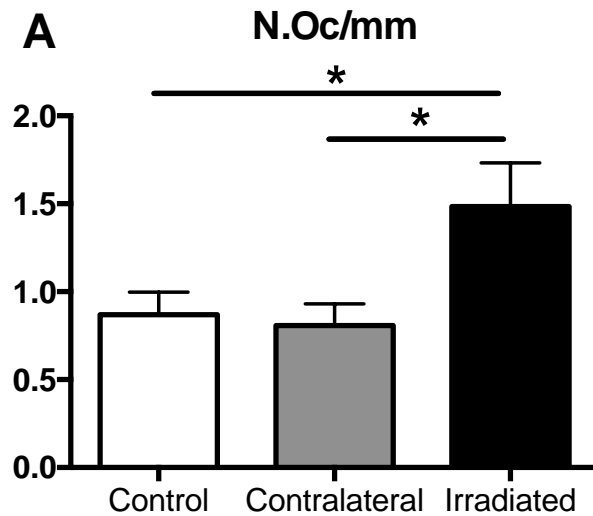


Figure 4. Single limb irradiation increases osteoclastogenesis and marrow adiposity in mice. Twenty-week male C57Bl/6 mice were irradiated (2Gy) at the right hindlimb and bone was collected, fixed and decalcified for histological analyses seven days post-irradiation and compared to age-matched, sham-irradiated control mice. (A) The number of osteoclast (N.Oc/mm) and (B) osteoblasts (N.Ob/mm) per unit of bone surface was quantified and (C) the total number of adipocytes were quantified in the marrow cavity in H&E stained sections (**p<0.01; ***p<0.001 by one-way ANOVA).

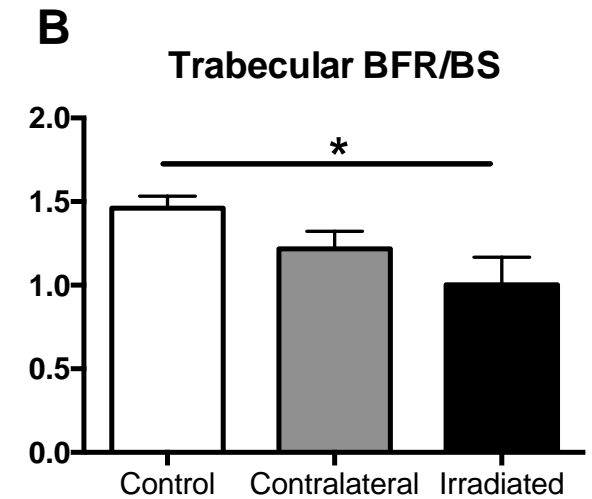
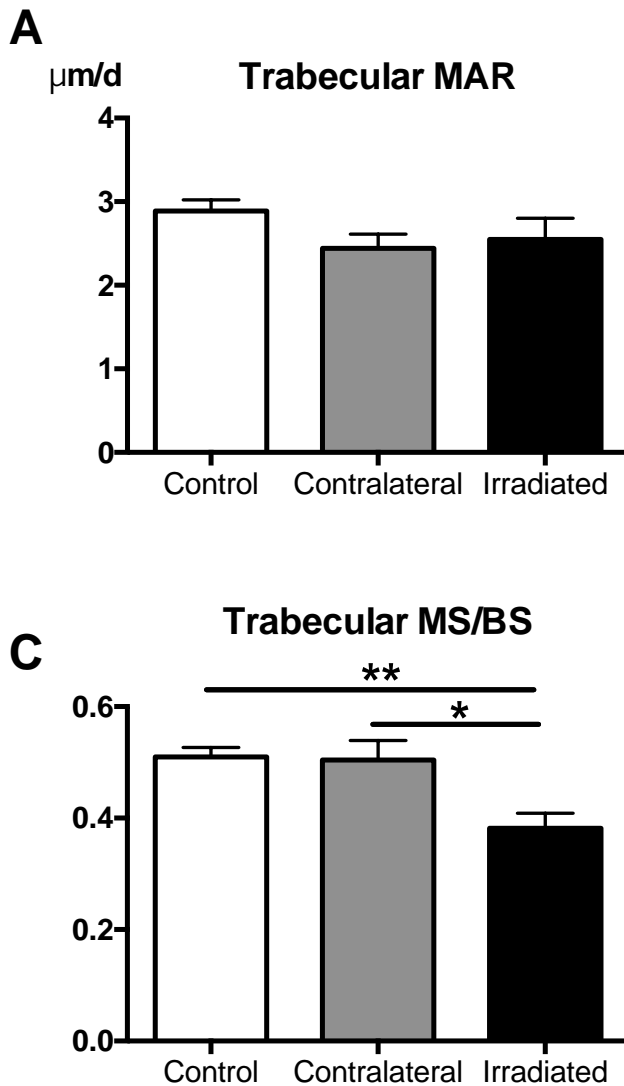


Figure 5. Single limb irradiation impaired trabecular bone formation and mineralization in mice. Twenty-week male C57Bl/6 mice were irradiated (2Gy) at the right hindlimb and seven days post-irradiation, bone was collected, fixed and embedded in plastic for histological analyses and compared to age-matched, sham-irradiated control mice. (A) Mineral apposition rate (B) mineral apposition rate relative to the total bone surface and (C) mineralized surface was quantified by dynamic histomorphometric analysis using the fluorescent double calcein labels (* $p < 0.05$; ** $p < 0.01$ by one-way ANOVA).

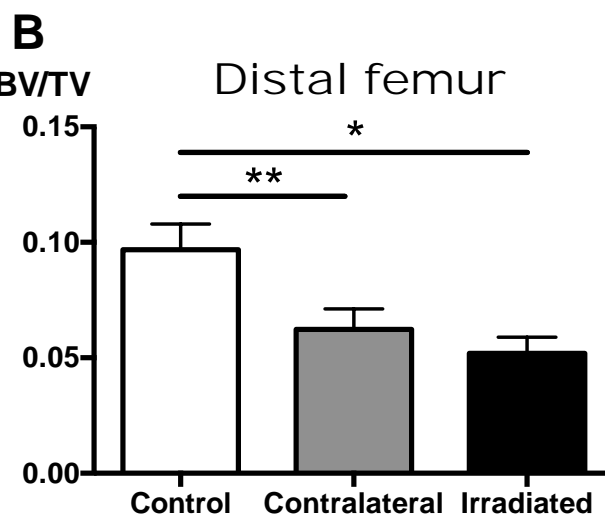
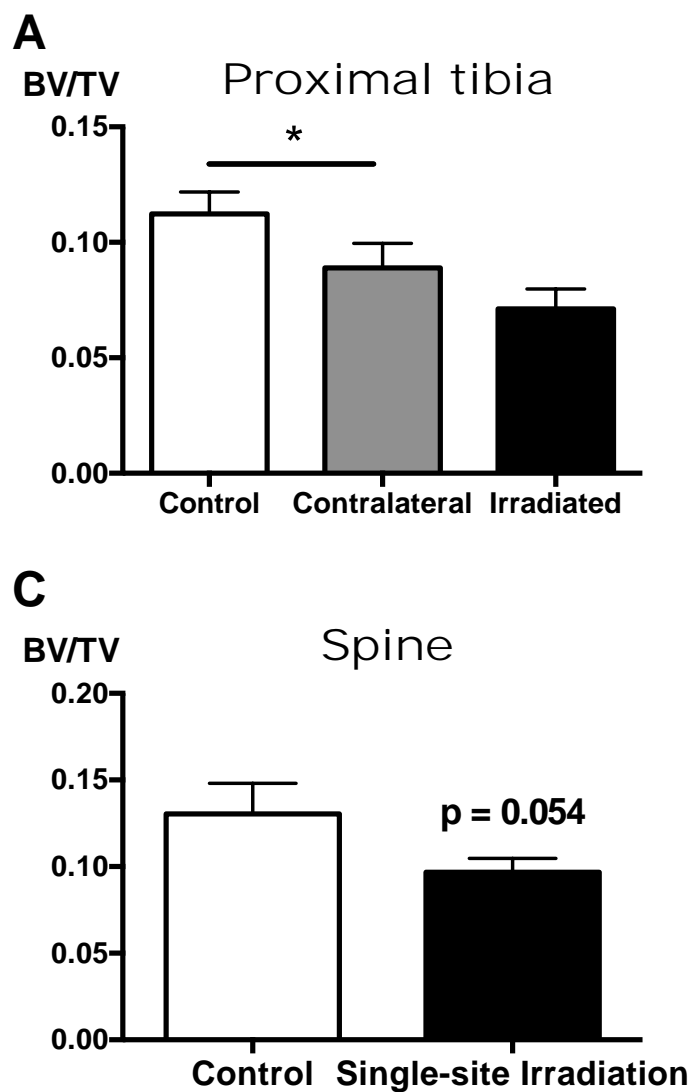


Figure 6. Single limb irradiation causes bone loss in young male athymic nude mice. Four-week athymic nude mice were irradiated (2Gy) at the right hindlimb and followed for 14 days for changes in bone volume fraction (BV/TV) at the (A) proximal tibia (B) distal femur and (C) spine by microcomputed tomography (* $p < 0.05$, ** $p < 0.01$ by one-way ANOVA).

KEY RESEARCH ACCOMPLISHMENTS

- *In situ* calvarial studies are complete and have provided a dose- and time-response for osteocyte apoptosis. Based on this work, we can also now definitively conclude that osteoblasts are less susceptible to radiation-induced DNA damage because the assays were performed head-to-head.
- An increase in osteoclast differentiation and activity following direct radiation exposure was confirmed *in vivo* and these new data align with previously presented *in vitro* data.
- We have found no evidence that osteocytes are the primary driver of radiation-induced osteoclastic bone destruction *in vivo* or *in vitro* and this finding has helped shape the direction of the remaining tasks.
- I obtained a \$20,000 internal grant from the Melvin and Bren Simon Cancer Center to use solely on the cancer center's Bio-plex® core laboratory. These funds were critical for the assessment of systemic cytokines in my radiation model.
- Serum samples were assayed for changes systemic factors that are known to affect bone following single-limb irradiation. We have found that key pro-inflammatory cytokines (IL-1, IL-6, IL-17, TNF- α) known to drive osteoclast-mediated bone loss did not change one week following radiation exposure and therefore the postulate that inflammation is driving radiation-induced bone loss can now be ruled out. Rather, we found that important anti-inflammatory, pro-bone formative cytokines (IL-3, IL-4, IL-10, RANTES/CCL2) were significantly decreased in irradiated mice relative to non-irradiated sham controls.
- Impaired bone mineralization was detected for the first time, and thus, we now have reason to examine osteoblast function by examining osteoid volume in histology slides that have previously been processed from our *in vivo* studies. This new finding will add to the mechanistic basis for radiation-induced bone loss.
- I have completed the necessary training required to execute the remaining studies in Tasks 7 & 8, including intra-cardiac inoculation of prostate cancer cells, X-ray lesion analyses, and tumor histology.
- The single limb irradiation model has been successfully reproduced in young Balb/c nu/nu athymic nude mice, and thus, prostate cancer bone metastasis studies have now commenced.
- In order to further my career development as an academic prostate cancer researcher, I joined the American Medical Writers Association (AMWA) and am utilizing membership resources to improve my science writing skills.

REPORTABLE OUTCOMES

Invited seminar:

- **Wright LE.** *Radiation-induced musculoskeletal defects.* Presentation of research for the National Institutes of Allergy and Infectious Diseases (NIAID) Medical Countermeasures Against Radiological Threats (MCART) site visit to Indiana University, Indianapolis, IN, August 14th 2012, hosted by the Department of Radiation Oncology.

Poster presentation:

- **Wright LE,** Buijs JT, John S, Peng Z, Harhash A, Waning D, Mohammad KS, Mendoca M, Chua HL, Wolfe H, Marks A, Orschell C, Guise TA. *Ionizing radiation induced both direct and systemic bone loss in murine models.* International Bone & Mineral Society's 12th International Conference on Cancer-Induced Bone Disease, Lyon, France, November 2012.

CONCLUSIONS

Effects of radiation on bone at the cellular level have not been comprehensively evaluated until now. We have demonstrated that osteocyte apoptosis can be induced by radiation exposure at a dose of 6Gy or higher on day seven post-irradiation. These studies have also revealed that osteoblasts are less susceptible to radiation-induced apoptosis yet the osteoblast may experience functional changes as a result of irradiation that impact the formation of mineralized bone. Thus far, we have found no evidence that irradiated osteocytes directly stimulate osteoclast formation, however, results previously presented from studies resulting from this grant showed that conditioned media collected from irradiated bone marrow (BM) cells stimulated osteoclastogenesis, indicating that soluble factors released from BM cells may contribute to radiation-induced bone loss. Direct irradiation of osteoclast precursor cells caused an increase in osteoclast activity both *in vitro* and *in vivo*. These data are important because they suggest that radiation-induced bone loss is largely driven by an increase in osteoclastic bone resorption, and these results could potentially be used to support the use of bisphosphonates, which target the osteoclast, in cancer patients undergoing radiation therapy. Effects of radiation on bone mineralization

were also detected in trabecular bone at one week following irradiation in our models, suggesting that while bone resorption is the main cause of bone loss, deleterious effects on bone formation may also contribute to bone loss and increased fracture risk in patients undergoing radiation therapy. A clinically relevant finding from our studies involves the repeated detection of bone loss at non-irradiated skeletal sites. These previously unreported results suggest that systemic effects of focal irradiation may also be contributing to the bone phenotype in cancer patients.

Taken together, these descriptive and mechanistic studies are the first of their kind to provide a complete picture of the effects of radiation exposure on bone. Understanding the mechanisms of osteoclast activation and bone loss in cancer patients could potentially lead to rapid implementation of therapies that would not only improve quality of life, but could also decrease morbidity and mortality after cancer treatment. We have now established the single-limb model of radiation-induced bone loss in athymic nude mice and are currently evaluating whether radiation insult to the bone microenvironment can increase the progression of prostate cancer bone metastases. Cancer mortality is typically not caused by growth of the primary tumor, but rather is the result of cancer metastasis to secondary sites, including the skeleton. The studies remaining in this training proposal will therefore evaluate the effects of radiation exposure on prostate cancer metastasis to bone in a murine model.

LIST OF PERSONEL RECEIVING PAY FROM RESEARCH EFFORT

- Laura E. Wright, Ph.D. (PI)

REFERENCES

- 1) Salvesen GS. Caspases: opening the boxes and interpreting the arrows. *Cell Death Differ* 2002; 9: 3–5.
- 2) Ghavami S, Hashemi M, Ande SR, Yeganeh B, Xiao W, Eshraghi M, Bus CJ, Kadkhoda K, Wiechec E, Halayko AJ, Los M. Apoptosis and cancer: mutations within caspase genes. *J. Med. Genet* 2009; 46: 497–510.
- 3) Koopman G, Reutelingsperger CP, Kuijten GAM, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* 1994; 84: 1415–20.
- 4) Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis—flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. *J Immunol Methods* 1995; 184: 39–51.
- 5) Constantino PD, Freidman CD, Steinberg MJ. Irradiated bone and its management. *Otolaryngol Clin North Am* 1995; 28: 1021-38.
- 6) Jegoux F, Malard O, Goyenvallée E, Aguado E, Daculsi. Radiation effects on bone healing and reconstruction interpretation of the literature. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2010; 109: 173-84.
- 7) Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, Ito M, Takeshita S, Ikeda K. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metabolism* 2007; 5: 464-75.
- 8) Kogianni G, Mann V, Noble BS. Apoptotic bodies convey activity capable of initiating osteoclastogenesis and localized bone destruction. *J Bone Miner Res* 2008; 23: 915-27.
- 9) Willey JS, Livingston EW, Robbins ME, Bourland JD, Tirado-Lee L, Smith-Sielicki H, Bateman TA. Risedronate prevents early radiation-induced osteoporosis in mice at multiple skeletal locations. *Bone* 2009; 44: 101-11.
- 10) Willey JS, Lloyd SA, Robbins ME, Bourland JD, Smith-Sielicki H, Bowman LC, Norrdin RW, Bateman TA. Early increase in osteoclast number in mice after whole-body irradiation with 2 Gy X rays. *Radiat Res* 2008; 170: 388-92.
- 11) Yang J, Shah R, Robling AG, Templeton E, Yang H, Tracey KJ, Bidwell JP. HMGB1 is a bone-active cytokine. *J Cell Physiol.* 2008; 214: 730-9.
- 12) Bentzen SM. Preventing or reducing late side effects of radiation therapy: radiobiology meets molecular pathology. *Nat Rev Cancer* 2006; 6: 702-13.
- 13) Konski A, Sowers M. Pelvic fractures following irradiation for endometrial carcinoma. *Int J Radiat Oncol Biol Phys* 1996; 35: 361-7.
- 14) Ikushima H, Osaki K, Furutani S, Yamashita K, Kishida Y, Kudoh T, Nishitani H. Pelvic bone complications following radiation therapy of gynecologic malignancies: clinical evaluation of radiation-induced pelvic insufficiency fractures. *Gynecol Oncol* 2006; 103: 1100-4.
- 15) Ogino I, Okamoto N, Ono Y, Kitamura T, Nakayama H. Pelvic insufficiency fractures in postmenopausal woman with advanced cervical cancer treated by radiotherapy. *Radiother Oncol* 2003; 68: 61-7.

- 16) Oh D, Huh SJ, Nam H, Park W, Han Y, Lim dH, Ahn YC, Lee JW, Kim BG, Bae DS, Lee JH. Pelvic insufficiency fracture after pelvic radiotherapy for cervical cancer: analysis of risk factors. *Int J Radiat Oncol Biol Phys* 2008; 70: 1183-8.
- 17) Noble BS, Peet N, Stevens HY, Brabbs A, Mosley JR, Reilly GC, Reeve J, Skerry TM, Lanyon LE. Mechanical loading: biphasic osteocyte survival and targeting of osteoclasts for bone destruction in rat cortical bone. *Am J Physiol Cell Physiol* 2003; 284: C934-C943.

APPENDICES

Abstract from the Cancer-Induced Bone Disease annual meeting, Lyon, France, November 2012

Ionizing Radiation Induces Both Direct and Systemic Bone Loss in Murine Models

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It is increasingly recognized that patients undergoing radiation therapy for cancer are at increased risk of musculoskeletal complications including bone loss and fracture. Effects of radiation on skeletal muscle in cancer patients have not been well studied. Similarly, effects of high dose rate radiation on the musculoskeletal system such as that from radiation accidents or dirty bombs are unknown. A total body irradiation (TBI) model was used to evaluate changes in bone, cytokine levels and skeletal muscle function. Female C57BL/6 mice (n=3-6/group) were irradiated (LD50/30; 800cGy, 137Cs, 62cGy/min) and followed for 8 weeks for changes in bone at the proximal tibial metaphysis by micro-computed tomography (μ CT) using a SCANCO vivaCT40. Serum cytokine levels were assayed by Bio-Plex® Assay (Bio-Rad). Skeletal muscle function was assessed by measurement of maximal contractile force of the extensor digitorum longus (EDL) muscle. Relative to sham-irradiated controls, trabecular bone volume (BV/TV) decreased by 38%, 66% and 72% on weeks 1, 2 and 3 post-irradiation, respectively ($p<0.0003$ by t-test). Serum levels of the osteoclastogenic cytokines IL-1 β , IL-2, TNF- α and GM-CSF were elevated at weeks 1-3 post-TBI relative to control ($p<0.05$ by t-test). Maximal contractile force in the EDL was significantly increased in TBI mice relative to controls for 3 weeks following irradiation ($p<0.05$ by two-way ANOVA) but subsequently declined at later time points. In a separate study modeling radiotherapy, C57BL/6 mice (n=15/group) were irradiated with 200 cGy at the right hind limb, and bone was assessed at baseline and 7 days post-irradiation by μ CT. BV/TV in irradiated tibiae decreased by 22% relative to baseline ($p<0.0001$ by paired t-test). Interestingly, non-irradiated contralateral tibiae lost 16% BV/TV relative to baseline ($p=0.003$) while sham-control animals did not lose bone. These results suggest that radiation-induced bone loss may be partially driven by systemic factors, including osteoclastogenic cytokines. Further studies are necessary to determine the relative contribution of inflammatory cytokines on short- and long-term radiation-induced bone loss as well as the mechanism of change in skeletal muscle function following irradiation. These studies emphasize the sensitivity of the musculoskeletal system to radiation, and may be useful in developing therapeutics that can prevent musculoskeletal weakness following radiation exposure.